

# Importance of showing HBsAg and HBcAg positivity in the liver for better aetiological definition of chronic liver disease

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**SUMMARY** Serum and tissue hepatitis B virus (HBV) markers were compared in 35 alcoholic and 23 non-alcoholic subjects affected by chronic liver disease. Seventeen point one per cent of alcoholic and 21.7% of non-alcoholic subjects had HBV tissue markers, but not serum markers, for this virus. It is therefore concluded that showing the presence of HBV tissue markers permits a better aetiological definition of hepatitis B surface antigen (HBsAg) negative chronic liver disease, both in alcoholic and non-alcoholic subjects.

Determination of hepatitis B virus (HBV) antigens in the serum and in sections of liver biopsy tissues is widely practiced. This is useful for establishing the diagnosis of HBV infection and provides a better understanding of the complex natural history of this type of viral hepatitis.

In our laboratory we daily monitor hepatitis B surface antigen (HBsAg) and hepatitis B core antigen (HBcAg), and the availability of these data prompted the idea of a comparison between the prevalence of these markers in serum and tissues, and the relation between them.

The first results were conflicting: in some cases serological and histological findings were in complete agreement,<sup>1-3</sup> but at the other times positivity was more often observed in the tissues than in the serum.<sup>4-6</sup>

From several studies it has emerged that patients with chronic liver disease often present with HBsAg or HBcAg, or both, in the liver, even in the absence of circulating HBsAg.<sup>5-12</sup> This is believed to be indicative of both a continuous "minimal" production of HBV and of its causative role in the aetiology of liver disease.<sup>13</sup>

In two recent studies the presence of HBsAg or HBcAg, or both, was in the liver—in the absence of the usual HBV serum markers—in patients with acute leukaemia undergoing antileukaemic treatment,<sup>14</sup> and in one non-alcoholic woman with chronic liver disease.<sup>15</sup> Similar findings in alcoholics with chronic

liver disease are quite rare. Brechot<sup>16</sup> observed serum HBV-DNA in five HBsAg negative alcoholics with anti-HBc or anti-HBs, or both, as well as in nine patients without any of the usual HBV markers. In a recent study undertaken by our centre<sup>17</sup> HBV tissue markers were observed in one alcoholic patient with chronic liver disease, all the other serum markers of HBV being negative. Such noticeable differences have not only prompted the need to verify the degree of specificity and sensitivity of methods presently used, but have also focused attention on the complex mode of reproduction of these viral particles.

With this in mind, the specific aim of our study was to contribute to a better aetiological definition of chronic liver disease, particularly when induced by alcohol abuse. We therefore studied a group of alcoholic patients affected by chronic liver disease, determining the presence of HBsAg and HBcAg in the liver and comparing tissue positivity with the pattern of HBV serum markers. Our control group comprised non-alcoholic subjects also suffering from chronic liver disease.

## Material and methods

### PATIENTS

We studied 35 alcoholic patients (two women, 33 men), mean age 55.8 years, with an alcohol consumption above 80 g/day (mean 144 g/day) for a period of at least 10 years. Each patient's personal history was carefully evaluated, particularly with

regard to negativity for intake of hepatotoxic drugs, previous hepatitis, and active homosexual contact. The histological diagnosis consisted of steatosis in four cases, chronic persistent hepatitis (CPH) in four, chronic active hepatitis (CAH) in 12, and cirrhosis in 15. None of the alcoholic patients presented all the features of alcoholic hepatitis, as defined by the International Group.<sup>18</sup> The histomorphological changes were very similar to those found in the "viral" type of chronic hepatitis, described in a recent study on liver histopathological changes in alcoholics.<sup>19</sup> The control group comprised 23 non-alcoholic patients (11 women, 12 men), mean age 52 years. The controls included patients with chronic liver disease, which was undoubtedly of the viral type. None of the controls had taken drugs liable to damage the liver, nor did they have a deficiency of  $\alpha_1$  antitrypsin (shown by the absence of periodic acid Schiff positive and diastase resistant granules in liver cells). The controls were comparable in age with the alcoholics and were taken from the same population sample. The histological diagnosis was CPH in four cases, CAH in six cases, and cirrhosis in 13.

#### SEROLOGY

All the sera were tested for the presence of HBsAg, anti-HBs, anti-HBc, HBeAg, and anti/HBe by radioimmunoassay using commercial kits (Abbott Laboratories, Chicago, Illinois). A multichannel autoanalyser was used to determine serum activities of alanine transferase, aspartate transferase, and  $\gamma$ -glutamyltransferase. The mean cell volume was determined with a Coulter counter, and the IgA values by the nephelometric method.

#### IMMUNOHISTOLOGY

Liver biopsy specimens, obtained during laparoscopy by means of biocut, were immediately treated by standard histological methods for the assessment of the varying histological conditions. HBsAg and HBcAg

intrahepatocellular determination was done by immunohistochemical methods, using immunoperoxidase (PAP method according to the Sternberger technique).<sup>20</sup> Test materials for showing the presence of HBsAg and HBcAg are included in Dako PAP Kit System K511 and the Dako PAP Kit System K523, respectively. The hepatic specimens were dewaxed, hydrated, and then incubated for 20 minutes in methanol and  $H_2O_2$  at 12 m/v to block hepatocellular endogenous peroxidases. The examination then proceeded at room temperature as follows:

- 1 Rinsing in water (five minutes).
- 2 Incubation in normal swine serum (20 minutes).
- 3 Rinsing in Tris buffer at pH 7.2-7.6 (five minutes).
- 4 Incubation with immunoglobulin fraction of rabbit antiserum to HBsAg and HBcAg (20 minutes).
- 5 Rinsing in Tris buffer (five minutes).
- 6 Incubation with immunoglobulin fraction of swine antiserum to rabbit immunoglobulin (20 minutes).
- 7 Rinsing in Tris buffer (five minutes).
- 8 Incubation in PAP (soluble horseradish peroxidase—rabbit antihorseradish peroxidase) complex (20 minutes).
- 9 Rinsing in Tris buffer (five minutes).
- 10 Incubation with DAB (3-3'-diamino-benzidine-tetrahydrochloride (20 mg) in 40 cc of Tris buffer and  $H_2O_2$  at 35 m/v (20  $\mu$ l) (20 minutes in a dark room).
- 11 Rinsing in water.
- 12 Staining with hematoxylin and eosin, periodic acid Schiff (PAS), phosphotungstic acid and Gridley stain.

To verify the specificity of the method the material was tested by: (i) incubation with rabbit anti-HBs serum absorbed with HBsAg (Dako Pap Kit System K511); (ii) substituting rabbit anti-HBs serum with normal HBsAg negative human serum; (iii) using normal liver tissue as control; (iv) incubation with rabbit anti-HBc serum absorbed with HBcAg (Dako Pap Kit System K523); (v) substituting rabbit anti-HBc

Table 1 Mean (SD) laboratory data in 35 alcoholic and 23 non-alcoholic patients

		Alcoholic patients				Non-alcoholic patients		
Normal values		Fatty liver (n = 4)	Chronic persistent hepatitis (n = 4)	Chronic active hepatitis (n = 12)	Cirrhosis (n = 15)	Chronic persistent hepatitis (n = 4)	Chronic active hepatitis (n = 6)	Cirrhosis (n = 13)
Aspartate transferase	0-30 U/l	45.8 (24.3)	53.8 (9.2)	62.7 (21.1)	50.3 (19.6)	40.3 (19)	115 (33.1)	53.8 (23.1)
Alanine transferase	0-30 U/l	62.0 (14.6)	42.0 (10.1)	54.7 (25.6)	33.8 (17.8)	24.3 (10.3)	122 (75.1)	54.2 (27.7)
Aspartate transferase/ alanine transferase		0.73 (0.32)	1.32 (0.25)	1.84 (0.91)	1.85 (1.08)	1.93 (0.89)	1.15 (0.69)	1.16 (0.49)
$\gamma$ -glutamyltransferase	0-28 U/l	17 (6)	39.5 (21.3)	187 (40)	88 (35)	60 (20)	44.5 (8.5)	40 (10)
IgA	50-370 mg/dl	269.7 (70)	356 (126)	563 (229)	702 (206)	394 (122)	249 (81)	396 (127)
Mean cell volume	77-91	93.1 (6)	92.8 (6.6)	97.8 (5.1)	93.2 (7.3)	96 (7)	95 (5.3)	94 (6.1)

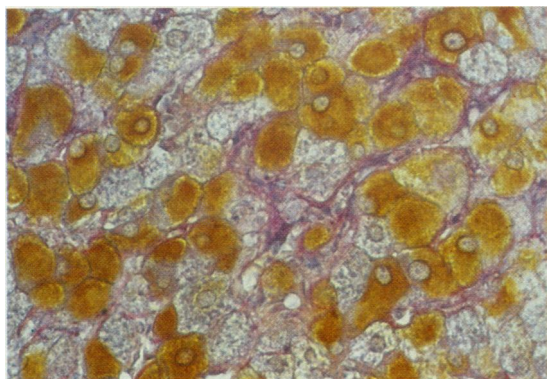


Fig 1 Liver from alcoholic patient with chronic active hepatitis showing cytoplasmic hepatitis B surface antigen (HBsAg). (Indirect immunoperoxidase staining for HBsAg.)  $\times 400$ .

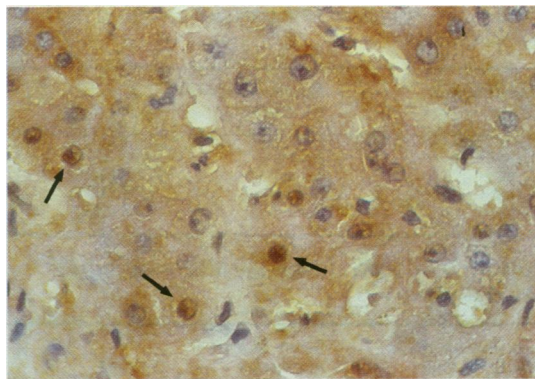


Fig 2 Hepatitis B core antigen (HBcAg) expression in nuclei of hepatocytes in alcoholic patient with chronic active hepatitis. (Indirect immunoperoxidase staining for HBcAg.)  $\times 280$ .

serum with normal HBcAg negative human serum; (vi) using normal liver tissue as control.

## Results

Table 1 shows the laboratory data for the two groups.

At least one HBV serum marker was found in 17 of the 35 alcoholic (48.6%) and 11 of the 23 non-alcoholic patients (47.8%). These figures are higher than that found (22%) in 8000 subjects from the same area as our patients and tested in the Blood Transfusion Centre at the Ospedale Avellino.

HBsAg or HBcAg, or both was found in liver cells in 16 of the 35 alcoholic subjects (45.7%) and in nine of the 23 non-alcoholic subjects (39.1%) (figs 1 and 2). Taking into account the presence of HBV serum and tissue markers, the prevalence of viral markers went up to 65.7% (23 of 35) of alcoholic patients and 69.6% (16 of 23) of non-alcoholic patients.

Tables 2 and 3 show the HBsAg and HBcAg tissue distribution in relation to the pattern of serum markers in alcoholics and non-alcoholics, respectively. Tables 4 and 5 show the distributions of serum and tissue positivity in alcoholics and non-alcoholics, respectively.

Table 2 HBV tissue markers and their association with HBV serum markers in 35 alcoholic patients

Serological patterns	Steatosis (n = 4)		Chronic persistent hepatitis (n = 4)		Chronic active hepatitis (n = 12)		Cirrhosis (n = 15)	
	HBsAg	HBcAg	HBsAg	HBcAg	HBsAg	HBcAg	HBsAg	HBcAg
HBsAg + anti-HBc	—	—	1	—	5	1	2	—
Anti-HBs + anti-HBc	—	—	—	—	—	—	—	—
Anti-HBc	—	—	1	—	1	1	—	—
No HBV serum marker	—	—	—	1	1	1	3	2

Table 3 HBV tissue markers and their association with HBV serum markers in 23 non-alcoholic patients

Serological patterns	HBV tissue markers					
	Chronic persistent hepatitis (n = 4)		Chronic active hepatitis (n = 6)		Cirrhosis (n = 13)	
	HBsAg	HBcAg	HBsAg	HBcAg	HBsAg	HBcAg
HBsAg + anti-HBc	1	—	—	—	3	1
Anti-HBs + anti-HBc	—	—	—	—	—	—
Only anti-HBc	—	—	—	—	—	—
No HBV serum marker	—	—	1	1	4	2

Table 4 Distribution of serum and tissue positivity in 35 alcoholic subjects

Liver HBsAg or HBeAg, or both	Serum at least one marker	No (%)
Tissue +	Serum +	10/35 (28.6)
Tissue -	Serum -	12/35 (34.3)
Tissue +	Serum -	6/35 (17.1)
Tissue -	Serum +	7/35 (20.0)

Table 5 Distribution of serum and tissue positivity in 23 non-alcoholic subjects

Liver HBsAg or HBeAg, or both	Serum at least one marker	No (%)
Tissue +	Serum +	5/23 (21.7)
Tissue -	Serum -	7/23 (30.4)
Tissue +	Serum -	5/23 (21.7)
Tissue -	Serum +	6/23 (26.1)

## Discussion

It has been shown that the association between alcohol abuse and HBV infection is severely damaging for the liver.<sup>21-22</sup> Several studies have reported the presence of HBV serum markers in alcoholics with chronic liver disease.<sup>23-26</sup> Nonetheless, HBV serum markers, although more common in this group than in the population at large, mainly consist of anti-HBs or anti-HBc, or both. Generally, the presence of chronic infection induced by HBV is indicated by the presence of circulating HBsAg or, in a limited number of cases, by the very high values of anti-HBc.<sup>27-28</sup> The results of this study show that 17.1% of alcoholics and 21.7% of non-alcoholics had HBV markers in the liver in the absence of any serological marker. The incidence of this tissue-serum discrepancy was similar between alcoholics and non-alcoholics. This series of patients was not tested for serum or liver tissue HBV-DNA. Our tissue positive, seronegative patients, alcohol abusers, and non-abusers, however, probably correspond to the patients with HBV-DNA but not serum markers, described by Nalpas *et al.*<sup>16</sup>

As already stated,<sup>14-17</sup> the negativity of HBV serum markers is not itself sufficient to exclude HBV infection, but could have a relevant role in the progression of chronic liver disease, even if the usual activity markers (HBsAg and HBeAg) are not observed.

Numerous hypotheses have been formulated to explain the discrepancy between serum negativity and tissue positivity for HBV markers. Incidentally, every hypothesis—depending on the case—may, indeed, be valid, in that each hypothetical factor might have had a causal or cocausal role. There are various factors which have been suggested:

- 1 Non-specific positivity of immunoperoxidase.<sup>29</sup>
- 2 An incomplete blockage of the increased hepatocellular endogenous peroxidase activity.<sup>30</sup>
- 3 Extremely low serum values of HBsAg.<sup>28 31-34</sup>
- 4 A masking of the serum antigens in immunocomplexes whenever antibodies are present.<sup>31 33</sup>
- 5 Modifications of the humoral immune response in the host.<sup>16</sup>
- 6 Uncoordinated relations between biological rhythms of viral reproduction, histological activity of the hepatic process, and elimination of the virus from infected cells.<sup>30</sup>
- 7 Multiplication of HBV in hepatic cells without release into the serum of antigens in concentrations which may be assessed by radioimmunoassay.<sup>6</sup>

The demonstration of infection induced by HBV when the usual serological markers are negative is particularly important in those subjects in whom both the personal history and laboratory investigations could indicate liver disease caused by alcohol abuse, thereby redefining the role of alcohol in determining chronic liver disease in alcoholics. Furthermore, not only does it permit attribution of a definitive aetiology to HBsAg negative liver disease in non-alcoholic subjects, but is also invalidates, to a great extent, the classic distinction between HBsAg positive and HBsAg negative chronic hepatitis.

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